Construction and Characterization In Vivo of

Bordetella pertussis aroA Mutants

MARK ROBERTS, DUNCAN MASKELL,† PAVEL NOVOTNY, AND GORDON DOUGAN*

Department of Molecular Biology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, England

Received 9 October 1989/Accepted 1 December 1989

A DNA fragment encoding a kanamycin resistance determinant was used to insertionally inactivate the cloned aroA gene of Bordetella pertussis in Escherichia coli K-12, and a conjugative shuttle vector system based on the suicide vector pRTP1 was used to deliver the mutations from E. coli back into B. pertussis CN2992FS and BPI. The aroA mutation was introduced by allelic exchange into the chromosome of B. pertussis, resulting in otherwise isogenic parental and aroA mutant pairs. The B. pertussis aroA mutants grew well on laboratory medium supplemented with aromatic compounds but failed to grow on unsupplemented medium. The B. pertussis aroA mutants expressed the normal B. pertussis extracellular, virulence-associated proteins; inactivated, whole-cell vaccines prepared from the mutants protected mice as efficiently as vaccines made from the parent strains against intracerebral challenge with the virulent B. pertussis 18323. Live B. pertussis aroA bacteria inefficiently colonized the lungs of NIH/S mice after they were challenged with aerosol, unlike the wild-type B. pertussis organism. Mice exposed to three separate aerosols of live B. pertussis aroA bacteria were protected against lung colonization after being exposed to an aerosol containing the virulent parental B. pertussis strain. High-level antibodies against B. pertussis rapidly appeared in the sera of mice immunized by aerosol with the B. pertussis aroA strains and challenged with the virulent parent.

Live vaccines based on attenuated microorganisms are effective in stimulating protective immune responses against a variety of pathogens. In the past, attenuated vaccine strains were derived empirically and the genetic changes responsible for attenuation were consequently unknown. This led to problems with the stability and quality control of certain live vaccines. Modern molecular genetic techniques can now be applied to construct stable, genetically defined, attenuated bacterial strains whose suitability for use as live vaccines in particular animal hosts can be assessed.

Attenuation can be achieved in a number of ways. One approach is to identify virulence determinants produced by the pathogen and construct strains, using genetic manipulation, which fail to produce those determinants. An example is the construction of strains of Vibrio cholerae, which do not express cholera enterotoxin (15, 22). When attenuated bacterial strains are constructed, it is essential to ensure that only defined, nonreverting mutations are introduced into the genome of the pathogen. In the case of V. cholerae, mutations in the enterotoxin were initially constructed in a cloned enterotoxin gene in Escherichia coli K-12 and the mutated gene was reintroduced into V. cholerae by using genetic crosses (15, 22).

An alternative approach to achieving attenuation is to introduce mutations into a key metabolic pathway whose function is essential for bacteria to survive and grow sufficiently in vivo to cause disease. Virulent Salmonella strains can be attenuated by introducing stable auxotrophic mutations affecting important metabolic pathways, including the prechorismate (aro mutants) and the purine biosynthetic pathways (13, 28). Salmonella strains harboring mutations in aroA (13, 19), aroC (7), aroD (23), or combinations of these mutations (7, 23) are attenuated and highly effective live oral vaccines against salmonellosis in several animal hosts (13, 19, 33). Salmonella spp. are invasive pathogens that can enter and grow inside eucaryotic cells (9, 10). At present, it is unknown whether aro mutants of noninvasive pathogens are attenuated.

Bordetella pertussis is the causative agent of whooping cough in humans. B. pertussis colonizes the human host by attaching to the ciliated epithelial cells lining the upper respiratory tract. There, the bacteria grow and express a variety of virulence-associated extracellular and surface-located proteins which induce disease symptoms. There have been reports that B. pertussis can enter eucaryotic cells, but in the human infection, B. pertussis is thought to predominantly colonize the mucosal surface of the respiratory tract (2, 8, 17). We recently reported the cloning in E. coli and sequencing of the B. pertussis aroA gene (20). As a preliminary step towards constructing rationally attenuated strains of B. pertussis and other Bordetella species, we have constructed B. pertussis aroA mutants and tested their abilities to colonize the lungs of experimentally infected mice. The results of the study are described in this report.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. B. pertussis CN2992F is a derivative of CN2992, the Wellcome whole-cell vaccine strain, which is modulated not by increased concentrations of nicotinic acid but by temperature only (21). A spontaneous streptomycin-resistant mutant of CN 2992F (2992FS) was used in this study. B. pertussis BPI, a streptomycin-resistant derivative of strain Tohama 1, was obtained from G. Miller, Stanford University, Palo Alto, Calif. E. coli AB2829 aroA was obtained from B. Bachmann.
pertussis 58, Ion A pertussis England. Plasmid pBPTaroA52 consists of a pUC18 vector containing a 2.2-kilobase-pair PstI fragment encoding the B. pertussis aroA gene (20).

B. pertussis was routinely grown in Stainer-Scholte (SS) liquid medium at 37°C (35) or on Cohen-Wheeler (CW) solid medium (4) containing 10% sterile defibrinated horse blood. B. pertussis aroA mutants were grown on CW blood agar with or without aromatic substituents (aromix) or in SS medium with or without aromix. Aromix stock contained 4 mg of tryptophan, 4 mg of tyrosine, 4 mg of phenylalanine, 1 mg of dihydroxybenzoic acid, and 1 mg of para-aminobenzoic acid per ml and was used as a 1-in-100 dilution in supplemented media. E. coli strains were routinely cultured on L agar or in L broth (18). Antibiotic concentrations for E. coli strains were as follows: ampicillin, 100 μg/ml; and kanamycin, 40 μg/ml. For B. pertussis, concentrations were as follows: kanamycin, 50 μg/ml; and streptomycin, 200 μg/ml.

DNA manipulations. Restriction endonucleases and T4 DNA ligase were obtained from Boehringer (Lewes, England) or GIBCO BRL (Paisley, Scotland) and were used according to the instructions of the manufacturers. DNA polymerase 1 large fragment (Klenow enzyme) was fast protein liquid chromatography pure from Pharmacia.

DNA preparations and hybridization. Plasmid DNA was purified by using standard techniques (18). B. pertussis chromosomal DNA was prepared by using a modification (19) of the method of Hull et al. (14). DNA was transferred to nylon (Pall Biodyne) filters by the method of Southern (34). Radioactively labeled DNA probes were obtained, using nick-translation as described by Maniatis et al. (18).

Bacterial conjugation. Bacteria were grown on appropriate solid culture medium; E. coli was grown overnight, and B. pertussis was grown for 3 days. Bacterial growth was swept from the plates with Dacron swabs and suspended in phosphate-buffered saline (PBS), pH 7.2. The optical density of the suspensions at 650 nm was determined and adjusted to 0.4, and mating mixtures were set up by mixing B. pertussis and E. coli cells in the ratios 10:1 and 100:1 of B. pertussis to E. coli. These were immediately plated onto CW blood agar plates containing 10 mM MgCl2. The plates were incubated at 37°C in sealed jars for 4 h. After this time, the bacteria were swabbed from the plate into PBS and plated, for selection of transconjugants, onto CW blood agar plates containing appropriate antibiotics and aromix. Plates were incubated at 37°C for up to 5 days.

Aerosol infection. Groups of female NIH/S mice (11 to 14 g and approximately 3 weeks old) were placed in cages on a rotating carousel in a plastic exposure chamber as described previously (27). A bacterial suspension in PBS was prepared from 2- to 3-day-old cultures of B. pertussis grown on CW blood agar plates containing aromix as appropriate. The mice were exposed to an aerosol (generated from the bacterial suspension) of 2 × 10⁹ CFU in PBS by a Turret mouthpiece tubing operated by a System 22 CR60 high-flow compressor (Medic-Aid, Pagham, West Sussex, United Kingdom) giving a very fine mist at a dynamic flow of 8.5 liters/min. The generated mist was drawn through a chamber by a vacuum pump at a passage of ca. 12 liters of air per min, which maintained 70% relative humidity in the chamber. The exposure to aerosol lasted 30 min; a period of 10 min then allowed the chamber to clear.

The course of the infection was assessed by performing counts of viable bacteria in lungs. Groups of four mice were removed at intervals and killed by cervical dislocation, and their lungs were aseptically removed and homogenized in a Potter-Elvehjem homogenizer with 2 ml of PBS. Dilutions of the homogenate were spotted onto CW blood agar plates containing streptomycin (and aromix, where appropriate), and the number of CFU was determined for each set of lungs.

Preparation of whole-cell vaccines. Strains were grown on CW agar supplemented with 10% defibrinated horse blood (and aromix, where appropriate) for 72 h. The growth was washed off with PBS, and the suspensions were incubated for 24 h at 37°C in the presence of 0.25% Formalin. Formalization was terminated by centrifugal washing of the cells with PBS and suspending the pellets in PBS containing 1/1,000 merthiolate. Bacterial concentration (expressed as milligrams per milliliter [dry weight]) was determined spectrophotometrically at this stage. The final vaccines were stored at 4°C before potency determination. Potency determination was performed according to the World Health Organization requirements, using a frozen B. pertussis 18323 challenge strain as reported previously (26). NIH/S mice between 11 and 14 g were immunized intraperitoneally with corresponding dilutions of vaccines and challenged 14 days later intracerebrally. The results were computed by parallel line probit analysis, and the relative potency of vaccines was related to the British pertussis reference vaccine 66/84.

Adenylyl cyclase and pertussis toxin determination. Adenylyl cyclase activity was determined in whole cells by the method of Salomon et al. (31). The CHO cell assay was performed as described by Hewlett et al. (12), using known concentrations of pertussis toxin as reference.

Detection of filamentous hemagglutinin and 69-kilodalton outer membrane protein. Both filamentous hemagglutinin (FHA) and the 69-kilodalton outer membrane protein were detected in B. pertussis, using Western blotting (immunoblotting). Whole-cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16) and transferred to nitrocellulose by the method of Towbin et al. (38). Proteins were visualized by incubating the nitrocellulose with either a rabbit polyclonal anti-FHA serum (1:500 dilution) or a mouse monoclonal anti-69-kilodalton outer membrane protein antibody (1:10 dilutions of hybridoma culture supernatant), and then the appropriate anti-species immunoglobulins horseradish peroxidase conjugate and 4-chloro-1-naphthol were used as the substrate (Sigma, Poole, Dorset, United Kingdom).

Anti-B. pertussis antibodies. The murine serum antibody response to B. pertussis was measured by an enzyme-linked immunosorbent assay (ELISA), using formalized B. pertussis as antigen. Formalized B. pertussis (50 μl; 5 × 10⁶ CFU per ml in 0.1 M carbonate buffer [pH 9.6]) were absorbed onto 96-well flat-bottomed microtiter plates (Costar Corp., Cambridge, Mass.) by incubation at 4°C overnight. The wells were aspirated and washed three times with PBS containing 0.05% Tween 20 (PBS-Tween) and incubated with 100 μl of PBS-Tween-0.1% bovine serum albumin. The wells were washed and incubated with 50 μl of goat anti-mouse immunoglobulin horseradish peroxidase conjugate of appropriate class specificity (Sigma) diluted 1 in 1,000 in PBS-Tween was added. After incubation at 37°C for 2 h, the plates were washed and incubated for 20 to 30 min at
room temperature with 50 µl of substrate (0.04% o-phenylenediamine dissolved in phosphate-citrate buffer [pH 5.0]–24 mM citrate–64 mM disodium hydrogen phosphate containing 40 µl of hydrogen peroxide).

The reaction was terminated by the addition of 50 µl of 1 M sulfuric acid. Plates were read in a Titertek Multiscan MCC ELISA reader at 492 nm.

Sera were obtained from groups of four mice by cardiac puncture and pooled. Sera were serially diluted and assayed for anti-B. pertussis immunoglobulins of the immunoglobulin G (IgG), IgM, and IgA classes, using heavy-chain-specific antibody conjugates.

RESULTS

Construction of B. pertussis aroA mutants. In order to construct B. pertussis aroA mutants, the cloned B. pertussis aroA gene was first inactivated in vitro. pBPTaroA52 consists of a pUC18 vector containing a 2.2-kilobase-pair PstI fragment encoding the B. pertussis aroA gene (Fig. 1). This plasmid can complement the aroA lesion in E. coli K-12 AB2829, allowing the strain to grow on minimal medium in the absence of aronix (20). In order to create a single BamH I site in the aroA coding sequence, the BamH I site in the polylinker of pBPTaroA52 was removed by digestion with Xba I and EcoRI; the resultant single-stranded ends were then filled in with Klenow enzyme and blunt-end ligation, thus reconstructing the EcoRI site in the resulting plasmid, pBPTaroA53. Next, the kanamycin resistance cassette from pUC4K was cloned on a BamH I fragment into the remaining BamH I site in the aroA coding region of pBPTaroA53 to create plasmid pBPTaroA54. Inactivation of the aroA gene was checked by transforming E. coli AB2829 with pBPTaroA54; transformants could not grow on minimal medium unless it was supplemented with aroA. In preparation for returning the inactivated aroA gene to B. pertussis, the mutated aroA locus was removed from pBPTaroA54 on the suicide shuttle vector pRTP1 as follows. pBPTaroA54 was cleaved to completion with EcoRI and then partially with HindIII. A 3.6-kilobase-pair EcoRI–HindIII fragment containing the cloned DNA from pBPTaroA54, including the Km' cassette, was gel purified and ligated into pRTP1 previously cleaved with EcoRI and HindIII. E. coli transformants harboring the resultant plasmid were selected for resistance to Amp' and Km'. The resulting plasmid was called pBPTaroA55.

pRTP1 is mobilizable by sequences derived from RP4 on the chromosome of E. coli SM10 and does not replicate in B. pertussis because of the absence of the pir gene product, carried in SM10 on a lysogenic bacteriophage. Furthermore, pRTP1 encodes the S12 allele which confers streptomycin sensitivity on otherwise streptomycin-resistant bacterial strains. E. coli SM10(pBPTaroA55) was plate mated with B. pertussis BP1 or CN2992FS, and transconjugants were selected with CW blood agar plates containing streptomycin and kanamycin as selective antibiotics.

Any B. pertussis colonies appearing on the selective plates should be aroA mutants, since Km' should only be expressed if the wild-type aroA allele is replaced on the chromosome by the mutated aroA gene. This recombination may take place by two general mechanisms, one involving crossover between homologous structures of B. pertussis DNA at a single site within or flanking the aroA gene (which would result in insertion of the entire recombinant plasmid) and the other involving two regions separated by the site of the kanamycin resistance cassette insert (which would result in replacement of only the aroA allele) without vector integration. Because of the S12 allele on pRTP1, streptomycin in the medium selects against the single crossovers and thus selects against vector integration.

Km' and Sm' B. pertussis transconjugants were purified by being replated on CW medium with or without aroamix. Purified colonies grew very slowly or not at all on medium without aroamix but grew at a rate similar to that of wild-type B. pertussis on medium containing aroamix, giving rise to colonies which were similar in size to the parent strains. Some of these colonies were subcultured on CW blood agar plates and, after 72 h, the growth was washed off and inoculated into 200 ml of SS medium in a 500-ml conical flask and incubated at 37°C in an orbital shaker. These isolates could grow in the SS medium containing aroamix but not in nonsupplemented medium.

After some matings with BP1 as a recipient, some small
colonies which grew on aromix-supplemented CW blood agar but failed to grow on unsupplemented CW blood agar were isolated. These colonies would not grow in SS medium with or without aromix and were not studied further. A number of individual transconjugants which displayed the expected aroA phenotype were stored, and one mutant derived from BP1 and one mutant derived from CN2992FS were used in further studies.

**Phenotypic properties of *B. pertussis* aroA mutants.** Transconjugants capable of growth in aromix-supplemented SS medium multiplied at a rate comparable to that of the parental strains on subculturing. However, they differed from the parental strains in that they produced a brownish discoloration of the SS medium which was similar in hue to the brownish pigment produced in SS medium during growth of *Bordetella parapertussis*. Preliminary nuclear magnetic resonance analysis showed the presence of unidentified aromatic components in the spent medium of *B. pertussis* aroA mutants (B. Sweatman, Physical Sciences, Wellcome Research Laboratories, unpublished results). To date, this pigment has not been characterized further.

The mutants produced adenylate cyclase, pertussis toxin, FHA, and 69-kilodalton outer membrane protein in amounts comparable to those produced by the parent strains. To assess the production of protective cell components, the aroA mutants were compared with the parent strains for their abilities to act as killed whole-cell vaccines in mice against intracerebral challenge with *B. pertussis* 18323. Whole-cell vaccines prepared from three aroA mutants were highly protective. There was no statistical difference between the protection afforded by the killed aroA vaccines and that of the British pertussis reference vaccine 66/84 (data not shown).

**Genotypic properties of *B. pertussis* aroA mutants.** The site of insertion of the kanamycin cassette in the genome of BP1 aroA and CN2992FS aroA was checked by Southern blotting. Total chromosomal DNA was prepared from parental and mutant strains and digested with *Nco*I, and the fragments were transferred to nylon membranes after being electrophoretically separated. Membranes were probed with a radioactively labeled preparation of the 1.4-kilobase *Parl-NcoI* DNA fragment from pBPTaroA52. This fragment contains the entire aroA coding sequence plus 54 base pairs upstream of the translation initiation codon and 13 base pairs downstream of the stop codon. The results are shown for CN2992FS and CN2992FS aroA (Fig. 1). The probe hybridized to a 3.5-kilobase *NcoI* fragment in the parental strain and a 4.8-kilobase *NcoI* fragment in the aroA mutant. The increased size of the fragment corresponds to the insertion of the kanamycin resistance gene (Fig. 1). This was confirmed by using digestion with other restriction endonucleases (results not shown). BP1 aroA produced an identical hybridization pattern.

**Survival of *B. pertussis* aroA mutants in vivo.** The *B. pertussis* parent strains and aroA mutants were assessed for their abilities to colonize the lungs of mice. Adult outbred NIH/S mice were exposed to an aerosol generated from a suspension of between 2 × 10^10^ and 4 × 10^10^ CFU of *B. pertussis* bacteria per ml. This reproducibly led to seeding of the mouse lungs with 10^3^ to 10^4^ *B. pertussis* organisms. Survival of viable bacteria was followed by performing viable counts on homogenates of lungs removed from groups of four mice at intervals after the mice were exposed to aerosol. The results obtained with CN2992FS and CN2992FS aroA are shown in Fig. 2. BP1 and BP1 aroA behaved in a similar manner (results not shown). The parental strain, after an initial drop in numbers during the first day, multiplied to reach 10^4^ to 10^6^ CFU in the lung 11 days postchallenge (Fig. 2). Thereafter, the counts decreased gradually, but *B. pertussis* could still be detected in the lungs 32 days after infection. CN2992FS aroA did not efficiently colonize the mouse lung. The numbers of CN2992FS aroA bacteria present decreased daily after aerosol presentation until bacteria were cleared from the lungs between days 5 and 8 (Fig. 2). The aroA mutants are clearly highly attenuated for colonization of the lungs relative to the wild-type parent strains.

**Vaccination of mice with *B. pertussis* aroA.** To determine whether *B. pertussis* aroA strains could function as effective live respiratory vaccines, mice were immunized with CN2992FS aroA by exposure to a bacterial aerosol. The immunized mice and age-matched controls were then challenged 3 to 4 weeks later (when the mice were approximately 7 to 8 weeks old) with a nonlethal dose of the parental wild-type strain. Viable counts were performed on the lungs to determine whether immunization affected colonization or clearance of the wild-type strain. A single dose of 10^7^ to 10^9^ CFU of live CN2992FS aroA affected the course of infection with wild-type CN2992FS in the lungs of immunized mice compared with that in controls, but the difference was not pronounced. The counts were lower in the lungs of immunized mice, and the organism was cleared faster (data not shown).
shown). To enhance the protection observed, mice were given multiple doses of the attenuated strain. Mice were immunized three times with intervals of 3 days between the first and second doses and 4 days between the second and third doses. The means of the doses received, calculated by using groups of four mice, were $2.6 \times 10^3$ CFU in the first dose, $8.6 \times 10^3$ CFU in the second dose, and $1.2 \times 10^4$ CFU in the third dose. The CN2992FS aroA strain was not detected in the lungs of mice 11 days after the final inoculation. The immunized and control mice were challenged with CN2992FS 24 days after the third dose was administered.

The immunized mice cleared the challenge strain rapidly. At 3 days postchallenge, only one of the four mice examined had *B. pertussis* present in its lungs, and all subsequent mice examined were free from *B. pertussis* (Fig. 3). The control mice remained colonized for at least 21 days after being exposed to CN2992FS. It can also be seen from the control group that CN2992FS did not grow to levels as high as those in younger mice and was cleared earlier than it was in younger mice. Aerosol immunization with three doses of a *B. pertussis* aroA mutant clearly prevents subsequent normal colonization of the lungs of mice by wild-type organisms.

**Serum antibody response to B. pertussis.** Sera were collected from aerosol-immunized and control mice at intervals after respiratory challenge with 2992FS and assayed for *B. pertussis* antibodies by ELISA, using whole *B. pertussis* as antigen. A low level of anti-*B. pertussis* antibodies was detected in the sera of immunized, but not control, mice 10 days before challenge (Fig. 4). Anti-pertussis immunoglobulins appeared rapidly in the sera of immunized mice and reached high levels by the second week after challenge (Fig. 4). The anti-*B. pertussis* antibodies remained high for several weeks and then decreased, returning to low levels by day 46 postchallenge (Fig. 4). The rise in pertussis-specific serum antibodies in immunized mice correlates with the disappearance of the challenge organism from the lungs. In contrast, significant levels of anti-pertussis antibodies were only detected in the sera of control mice 46 days after challenge, which is 25 days after *B. pertussis* was last isolated from their lungs (Fig. 4).

The predominant class of serum anti-*B. pertussis* immunoglobulins, in both vaccinated and control mice, was IgG, but significant rises in pertussis-specific IgA and IgM were detected in both groups of mice.

**DISCUSSION**

In this report we describe the construction of *B. pertussis* strains with an insertion mutation in the *aroA* gene and the preliminary characterization of the mutants in vivo, using aerosol challenge of mice. Although *B. pertussis* is a fastidious organism, the *aroA* mutants grew well in laboratory medium supplemented with aromix. However, in the mouse challenge systems employed, the *aroA* strains were much less efficient at colonizing the lungs than were the wild-type parental strains. *B. pertussis* *aroA* strains are therefore attenuated for growth in vivo in our mouse lung colonization
After *B. pertussis* aroA mutants in vitro. mutants in protective wild-type with when used tion for virulence. poorly work tained in aerosol-immunized (0) mice after infection were diluted by ELISA, each group. This indicates that local factors are responsible for clearing bacteria from the lungs of unvaccinated mice. Geller and Pittman (11) detected *B. pertussis*-specific immunoglobulins, predominantly IgA, in the tracheobronchial washings of intranasally infected mice 15 days after infection. The only change in the serum immunoglobulin profile was a change in serum IgA (the specificity of which was not determined) 30 days after the initiation of infection. We are currently investigating the local immune response in the murine lung after administration of *B. pertussis* aroA.

Preliminary studies using convalescent sera of immunized and nonimmunized mice suggest that the antibody response is directed against several polypeptides (our unpublished results). The availability of purified *B. pertussis* antigens should make characterization of humoral and cellular immune responses to *B. pertussis* simpler.
The mouse aerosol challenge system is an unsatisfactory model for human whooping cough. We have never observed mouse-to-mouse lung infection with *B. pertussis*, although this is common with *Bordetella bronchiseptica*, which causes serious infections in a variety of animal species. We have recently constructed *B. bronchiseptica* aroA mutants, using methods similar to those described in this report. These will be evaluated in the appropriate model systems to see whether other *Bordetella* species, better adapted to survival in their hosts, can be attenuated by using aroA mutations.

*Salmonella* aroA mutants have been used to deliver heterologous antigens to the mammalian immune system. The heterologous antigens have been derived from bacteria (3, 19), viruses (37), and parasites (30). After the attenuated *Salmonella* species expressing the heterologous antigen have been delivered orally, both systemic and secretory antibody responses have been detected. The attenuated *B. pertussis* aroA strains described in this report could be used to deliver non-*Bordetella* antigens to the respiratory tract mucosal surface. We are currently investigating this possibility.

**LITERATURE CITED**


